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Survivin Silencing as a Promising Strategy To Enhance the Sensitivity of Cancer Cells to Chemotherapeutic Agents

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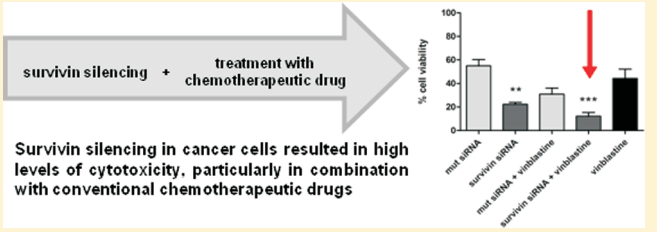
Supporting Information

ABSTRACT: Since clinical application of conventional cancer therapies is usually limited by drug resistance and toxic side effects, combination of chemotherapeutic agents with gene therapy appears as an attractive therapeutic strategy to overcome these issues. Being selectively expressed in tumor tissues, survivin is a promising target for the development of anticancer strategies aimed at eliminating tumor cells while sparing normal tissues. In this work, we achieved substantial protein knockdown in a number of human cell lines, namely, A549, HeLa and MCF-7 cells which overexpress survivin, after treatment with anti-survivin siRNAs, which was associated with a significant reduction of cell viability, when compared to treatment with control siRNAs. Interestingly, when the survivin-silencing approach was combined with a chemotherapeutic agent, an enhancement of the therapeutic effect was achieved. Treatment with anti-survivin siRNAs resulted in high levels of caspase 3/7 activation, and an enhancement of this effect was observed when survivin silencing was combined with vinblastine. In addition, we showed that for A549 and HeLa cells survivin silencing contributes to the reversion of cell resistance to doxorubicin. Overall, we demonstrate that the combination of a survivin-directed silencing strategy with chemotherapeutic agents constitutes a valuable approach for cancer treatment.



KEYWORDS: cancer gene therapy, survivin, chemotherapy, RNA interference, apoptosis, doxorubicin, vinblastine

INTRODUCTION

Cancer is a leading cause of death worldwide, being responsible for around 13% of all deaths each year. The discovery of new potential targets or improvement of existing therapies is therefore mandatory. Evasion of programmed cell death has been recognized as one of the six essential alterations in cell physiology that characterize malignant growth and is considered a hallmark of most types of cancer.[1,2] It has also been demonstrated that cancers possessing alterations in proteins involved in cell death signaling are often not only resistant to chemotherapeutic agents but also more difficult to treat using proapoptotic drugs.[1,3] Apoptosis, the most common and well-defined form of programmed cell death, is a natural process responsible for the clearance of unwanted cells, which plays an essential role in embryonic development and maintenance of tissue homeostasis.[1,4,5] However, deregulation of apoptosis disrupts the delicate balance between cell proliferation and cell death and has been implicated in numerous pathological conditions, including cancer.[4,6]

Two alternative pathways can initiate apoptosis: the extrinsic pathway, mediated by death receptors on the cell surface, and the intrinsic pathway, mediated by mitochondria.[5,7] In addition to the proteins that are directly involved in cell death signaling, two important families of apoptosis regulators have been identified: the Bcl-2 family, which comprises molecules with pro- or antiapoptotic functions, and the inhibitor of apoptosis proteins (IAPs), which include XIAP, c-IAP1, c-IAP2 and survivin.[1,8] IAPs have been shown to play a major role in apoptosis regulation by inhibiting the intrinsic and the extrinsic pathways of programmed cell death.[9] This class of proteins could be therefore described as being responsible for establishing a threshold at which caspases are kept inactive, while at the same time providing a pool of active caspases which can rapidly induce cell death when necessary.[10] The fact that IAPs can inhibit both pathways of controlled cell death renders this class of proteins particularly attractive to be targeted in cancer therapy. Survivin is a member of the IAP family of proteins that has an important role in two critical cellular processes, inhibition of apoptosis and cell proliferation.[11−20] Since this protein appears to be essential for both tumor cell proliferation and viability, and due to its overexpression in cancer cells and very low or absent expression in normal cells, survivin constitutes a particularly attractive tumor biomarker and a promising target for anticancer therapy.[11,21,22]

The ability of survivin to counteract proapoptotic stimuli has been shown to enhance cancer cell survival and render malignant cells resistant to conventional antitumoral treatments.[11,23−25] Therefore,

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strategies aimed at silencing survivin expression are expected to inhibit cancer cells' survival and enhance their sensitivity to conventional proapoptotic drugs.[24−26] In this study, we tested a strategy that combines gene therapy through survivin silencing using RNA interference technology, with conventional chemotherapeutic agents, namely, doxorubicin and vinblastine.

Our results demonstrate that survivin silencing sensitizes cancer cells to the cytotoxic action of conventional chemotherapeutic agents, which may represent an advance toward a targeted gene therapy to cancer with reduced side effects.

MATERIALS AND METHODS

Materials. The anti-GFP siRNA and the anti-survivin siRNA (sense, 5′GGACCACCGCAUCUCUACAdTdT3′; antisense, 3′dTdTCCUGGUGGCGUAGAGAUGU5′)[27] were obtained from Dharmacon (Lafayette, CO, USA). The control nonsilencing siRNA (mut siRNA) was obtained from Ambion (Austin, TX, USA). The Cy3-labeled nonspecific siRNA sequence was purchased from Ambion (Austin, TX, USA).

Doxorubicin and vinblastine were generously provided by the pharmaceutical services of the University Hospital of Coimbra (Portugal). Aliquoted stock solutions were stored at −20 °C.

Cells. HeLa cells (human epithelial cervical carcinoma), MCF-7 (human breast adenocarcinoma) and A549 cells (human epithelial lung adenocarcinoma) were maintained at 37 °C, under 5% CO_2, in Dulbecco modified Eagle medium—high glucose (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biochrom KG), and with 100 units of penicillin and 100 μg of streptomycin (Sigma) per mL.

For cell viability and caspase activity determination, cells were plated at a density of 0.5×10^5 cells/well onto 12-well plates. For QRT-PCR experiments, HeLa and A549 cells were plated at a density of 1×10^5 cells/well onto 6-well plates. In the case of flow cytometry experiments, HeLa and A549 cells were plated at a density of 0.6×10^5 cells/well onto 12-well plate for apoptosis rate determination through annexin-V/PI staining. Cells were plated twenty-four hours prior to incubation with siRNA complexes.

Cell Transfection and Incubation with Drugs. Complexes of siRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were prepared according to manufacturer's instructions.

Twenty-four hours after plating, cells were incubated with the siRNA/Lipofectamine complexes at the appropriate concentration (50 or 100 nM) in antibiotic- and serum-free OptiMEM (Invitrogen, Carlsbad, CA, USA), for four hours at 37 °C. After this incubation period, transfection medium was replaced with fresh medium containing 10% (v/v) FBS.

Cell treatment with the drugs, doxorubicin and vinblastine, was performed 48 h after plating. For the IC_{50} determination experiments, nontransfected cells were incubated with different concentrations of the drugs (0.01, 0.1, 0.5, 0.75, 1, 1.5, 2, and 5 μM doxorubicin and 0.01, 0.025, 0.05, 0.075, 0.1, 0.2, and 0.5 μM vinblastine) prepared in medium with 10% (v/v) FBS, for four hours at 37 °C. After this incubation period, drug-containing medium was replaced with fresh medium. For studies combining gene silencing treatment with chemotherapy, 24 h after transfection with the siRNA delivery systems, A549 and HeLa cells were incubated with doxorubicin or vinblastine at a concentration close to the IC_{50} (1 μM doxorubicin, for both cell lines, and 0.1 and 0.025 μM vinblastine for A549 and HeLa cells, respectively), as described above.

After transfection and/or incubation with the drugs, cells were analyzed for determination of viability, protein and mRNA levels, assessment of caspase activity and apoptosis, as described below.

Western Blot Analysis. Seventy-two hours after siRNA delivery, protein extracts were obtained from HeLa, A549 and MCF-7 cells using a lysis buffer (50 mM NaCl, 50 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma), 10 μg/mL DTT and 1 mM PMSF. Protein content was determined using the Bio-Rad protein assay (Bio-Rad), and 20 μg of total protein was resuspended in a loading buffer (20% glycerol, 10% SDS, 0.1% bromophenol blue), incubated for 5 min at 95 °C and loaded onto a 12% polyacrylamide gel. After electrophoresis, the proteins were blotted onto a PVDF membrane according to standard protocols. After blocking in 5% nonfat milk, the membrane was incubated with the appropriate primary antibody (anti-survivin 1:1000, Chemicon-Millipore, Billerica, MA, USA) overnight at 4 °C, and with the appropriate secondary antibody (1:20000) (Amersham, Uppsala, Sweden) for 2 h at room temperature. Equal protein loading was shown by reprobing the membrane with an anti-α-tubulin antibody (1:10000) (Sigma) and with the appropriate secondary antibody. After this incubation period, the blots were washed several times with saline buffer (TBS/T: 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween and 5 mg/mL nonfat powder milk) and incubated with ECF (alkaline phosphatase substrate; 20 μL of ECF/cm^2 of membrane) for 5 min at room temperature and then submitted to fluorescence detection at 570 nm using a VersaDoc Imaging System model 3000 (Bio-Rad). For each membrane, the analysis of band intensity was performed using the Quantity One software (Bio-Rad).

Extraction of RNA and cDNA Synthesis. RNA was recovered from A549 and HeLa cells, 72 h after cell transfection, using the MasterPure RNA Purification Kit (Epicenter Biotechnologies, Madison, WI, USA), according to the manufacturer's instructions, and then treated with DNase I. Briefly, cells were lysed using the tissue and cell lysis solution from the MasterPure RNA purification kit containing proteinase K, and cell lysates were collected to microcentrifuge tubes. MPC protein precipitation reagent was added to protein extract from the lysates. Then, 500 μL of isopropanol was added to the recovered supernatant to promote total nucleic acid precipitation, which was pelleted by centrifugation. Removal of contaminating DNA from the RNA was then performed by addition of a DNase I solution and carrying out another step of protein removal using MPC protein precipitation reagent. RNA was finally pelleted by centrifugation and rinsed twice with 70% ethanol, and resuspended in TE buffer. After quantification, RNA was converted to cDNA using the Superscript III first strand synthesis kit (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions.

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR). Quantitative PCR was performed in an iQ 5 thermocycler using 96-well microtiter plates and the iQ SYBR Green Supermix Kit. The primers for the target gene (survivin) and the housekeeping gene (HPRT-1) were predesigned by Qiagen (QuantiTect Primer, Qiagen). The percentage of survivin knockdown was determined by the ΔΔCt method, using HPRT-1 as housekeeping gene, according to the following formulas: ΔCt-(survivin siRNA) = Ct(survivin gene)−Ct(HPRT-1 gene); ΔCt(mut siRNA) = Ct(survivin gene) − Ct(HPRT-1 gene);

$\Delta\Delta$Ct = ΔCt(survivin siRNA) − ΔCt(mut siRNA); % knockdown =100% − ($2^{-\Delta\Delta Ct} \times 100$). The $\Delta\Delta$Ct value indicates the changes in RNA transcription caused by treatment with anti-survivin siRNAs, normalized to RNA transcription changes in cells treated with mut siRNAs.

Determination of Apoptosis Rate by Flow Cytometry. Apoptosis detection was performed using the ApopNexin FITC apoptosis detection kit for flow cytometry analysis (Chemicon-Millipore, Billerica, MA, USA). Forty-eight hours postincubation with doxorubicin, cells were collected after digestion with trypsin, washed with PBS three times, and resuspended in 500 μL of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cell staining with annexin V and PI was performed according to the manufacturer's instructions. FITC-labeled annexin V and PI were added to the cells, followed by 15 min incubation in the dark at room temperature. The apoptosis rate was immediately determined by flow cytometry. Bicolor analysis of cell suspensions was performed using the CellQuest software, according to the ApopNexin FITC apoptosis detection kit manufacturer's instructions.

Evaluation of Cell Viability. Cell viability was assessed under the different experimental conditions by a modified Alamar Blue assay, as described previously. Briefly, 48 and 72 h post-transfection, the cells were incubated with DMEM containing 10% (v/v) Alamar Blue dye. After 1 h incubation at 37 °C, the absorbance of the medium was measured at 570 and 600 nm. Cell viability was calculated, as a percentage of the nontransfected control cells, according to eq 1:

$$\text{cell viability (\% of control)} = [(A_{570} - A_{600})/(A\prime_{570} - A\prime_{600})] \times 100 \quad (1)$$

where A_{570} and A_{600} are the absorbances of the samples, and A'_{570} and A'_{600} those of control cells, at the indicated wavelengths.

Evaluation of Caspase-3/7 Activity. Cell extracts were prepared from HeLa and A549 cells, according to the manufacturer's instructions (SensoLyte Homogeneous AMC Caspase-3/7 assay kit, Ana Spec, Fremont, CA, USA). Briefly, cells were washed with ice-cold PBS and lysed with 1× lysis buffer, diluted from the 10× lysis buffer included in the assay kit. Cells were scraped off from the plates, and cell suspension was collected in a microcentrifuge tube. The cell suspension was rotated on a rotating apparatus for 30 min at 4 °C and then centrifuged at 2500*g* for 10 min at 4 °C. The supernatant was then collected and stored at −80 °C. Caspase 3/7 activity was measured 48 h post-transfection as the ability of cell extract to catalyze the cleavage of Ac-DEVD-AMC and release the AMC fluorochrome. Protein content of cell extracts was determined using the Bio-Rad protein assay (Bio-Rad). To initiate the enzymatic reaction, 50 μL of the cell lysate was transferred into the wells of a black 96-well plate (Costar, Cambridge, CA, USA), followed by the addition of 50 μL of assay buffer containing DTT and caspase-3/7 substrate. Reagents were mixed by shaking the plate in a shaker for 30−60 s at 100−200 rpm, and the fluorescence signal was continuously recorded every 30 min for 5 to 7 h. The plate was always maintained at 37 °C between and during measurements. Caspase-3/7 activity was determined by first plotting data as RFU/mg of protein versus time for each sample, and then determining the slope of the linear portion of the data plot.

Statistical Analysis. Results from all experiments are represented as mean ± standard deviation (SD). Data were analyzed using GraphPad Software, Inc., La Jolla, CA, USA. Statistical significances of differences between data were evaluated using ANOVA Tukey's multiple comparison test.

RESULTS

Evaluation of Survivin Silencing in Different Human Cancer Cell Lines. Initial studies were performed to determine the optimal siRNA concentration for survivin gene silencing. For this purpose, we examined the effect of anti-GFP and control (mut) siRNAs complexed with Lipofectamine 2000, delivered to HT 1080 GFP-expressing cells at the concentrations of 50 and 100 nM. A decrease in MFI levels of the population of GFP-expressing cells to one-third was observed at a 50 nM concentration of anti-GFP siRNAs, while a concentration of 100 nM reduced MFI to approximately one-fourth (data not shown). No decrease in the MFI levels was detected in cells treated with 50 nM mut siRNA, while a 15% reduction was observed upon cell incubation with 100 nM mut siRNA (data not shown), indicating that some unspecific gene silencing is occurring at this concentration. Therefore, subsequent experiments were performed using 50 nM siRNAs/well. For the studies on target validation involving gene silencing Lipofectamine 2000 was used as the siRNA delivery vector, since it has been demonstrated to efficiently and reproducibly transfect a broad range of cell lines in vitro.[28]

Treatment of A549, HeLa and MCF-7 cells with anti-survivin siRNAs complexed with Lipofectamine 2000 resulted in a pronounced effect on the viability of A549 (Figure 1 A) and HeLa cells (Figure 1 B), particularly 72 h after transfection, under which conditions only about 20% of viability was observed in HeLa cells. In A549 cells, the maximum reduction of viability was 55%, obtained 72 h after treatment with survivin siRNA (Figure 1 A). In contrast, such treatment did not result in any significant effect on the viability of MCF-7 cells (Figure 1 C). Although for HeLa cells treatment with survivin siRNAs has resulted in a higher reduction of viability than in A549 cells, the effect of the control treatment using mut siRNAs also resulted in a more pronounced decrease in cell viability (around 40%), as compared to that obtained for A549 cells (Figure 1 B). These nonspecific effects on cell viability may be due to off-target effects of siRNAs or to inherent toxicity of the delivery system. Noticeably, 48 h after transfection the reduction of cell viability obtained by survivin silencing was already significantly different from that observed with control siRNAs, in both A549 and HeLa cells (Figure 1A,B).

Survivin protein levels were determined in all three cell lines, and it was possible to confirm a decrease in survivin levels following the treatment with anti-survivin siRNAs, but not when using nonsilencing siRNAs (Figure 1D,E). However, since survivin knockdown in MCF-7 cells did not result in measurable effects on cell viability, this cell line was not used in further experiments.

To further confirm the specific gene silencing effect of the anti-survivin siRNA treatment, the determination of survivin mRNA levels was performed in A549 and HeLa cells (Figure 1 F). Approximately 80% reduction in survivin mRNA levels was observed in both cell lines treated with survivin siRNAs, when comparing to that using mut siRNAs, confirming that the reduction observed in protein levels was caused by survivin mRNA degradation.

Effect of the Combination of Chemotherapeutic Agents with Survivin Gene Silencing on Viability of Human Cancer Cells. Aiming at enhancing the cytotoxic activity of doxorubicin

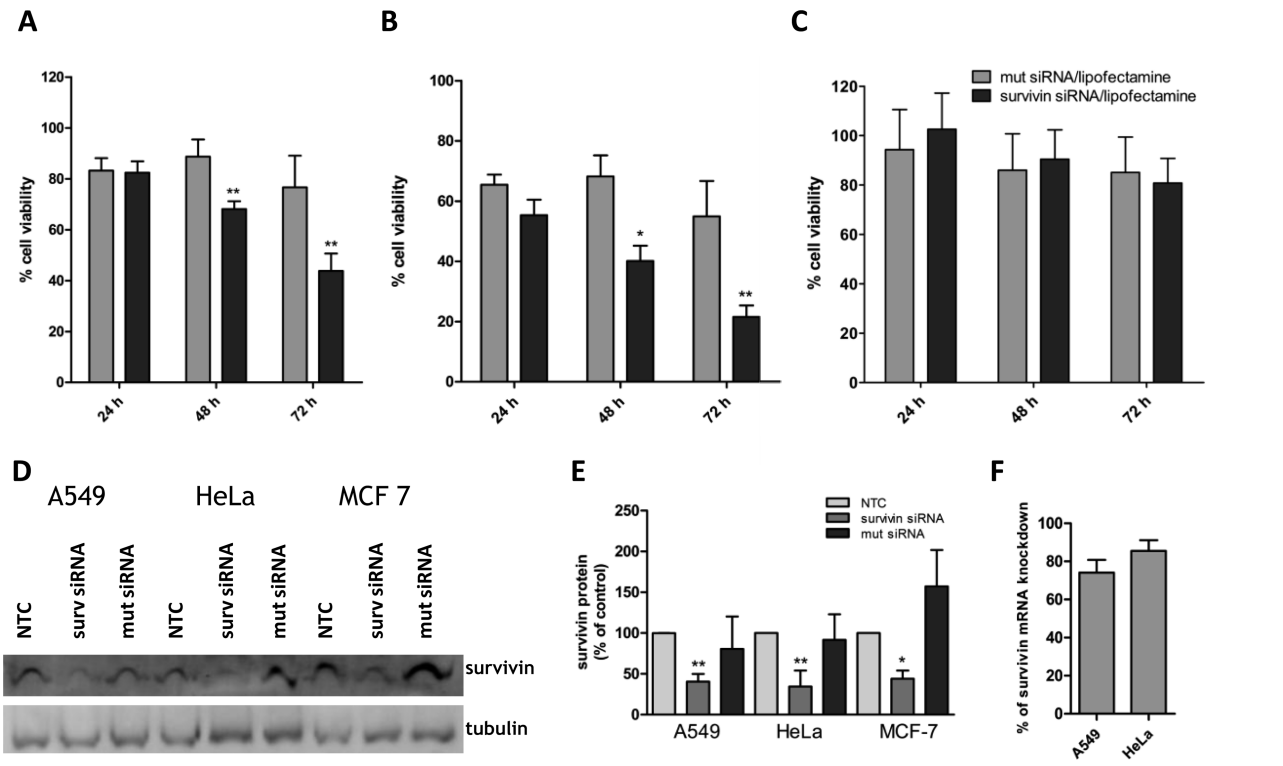

Figure 1. Evaluation of the effect of survivin silencing on cell viability, protein and mRNA levels. A549, HeLa and MCF-7 cells were incubated with anti-survivin or mut siRNA/Lipofectamine 2000 complexes, at a final siRNA concentration of 50 nM, for 4 h at 37 °C. Viability of A549 (A), HeLa (B) and MCF-7 (C) cells was evaluated by the Alamar Blue assay 24, 48, and 72 h post-transfection, and is expressed as the percentage of control (nontreated cells). To determine survivin protein levels after siRNA treatment, protein extracts were obtained from A549, HeLa and MCF-7 cells using a lysis buffer containing protease inhibitors and analyzed by Western blot (D and E). For determination of survivin mRNA levels, after equivalent treatment, RNA was recovered from A549 and HeLa cells and converted to cDNA, which was analyzed by quantitative PCR (F). The % of survivin knockdown was determined by the ΔΔCt method, as described in Materials and Methods, and indicates the changes in RNA transcription caused by treatment with anti-survivin siRNAs, normalized to RNA transcription changes in cells treated with mut siRNAs. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to treatment with mut siRNA at the same time points.

and vinblastine in A549 and HeLa cells, we assessed the effect of sequentially combining survivin silencing and treatment with doxorubicin or vinblastine. Preliminary studies were performed in order to establish the optimal conditions for the incubation time of cells with the drugs and the time points for cell viability measurements. These studies suggested that an incubation time of 4 h with the drugs was appropriate, and cell viability was assessed 24 and 48 h after incubation with the drugs since after 4 h incubation of cells with the drugs no differences in cell viability were observed among the different tested conditions and results obtained after 72 h incubation were very similar to those found after 48 h incubation (data not shown). Both A549 and HeLa cells (Supplementary Figure 1 in the Supporting Information) exhibited some degree of resistance to doxorubicin, 40% being the maximal cell death achieved over the drug concentration range from 0.5 to 5 μM. A concentration of doxorubicin of 1 μM was selected to treat both cell lines in further studies (Supplementary Figure 1 in the Supporting Information). The concentration of 0.1 μM vinblastine was chosen to treat A549 cells in combination with the gene silencing approach, and 0.025 μM was selected in the case of HeLa cells (Supplementary Figure 1 in the Supporting Information).

As observed in Figure 2, 48 h after transfection A549 cells sequentially treated with survivin siRNAs and doxorubicin or vinblastine presented a significantly lower viability than that observed following treatment with doxorubicin or vinblastine alone (Figure 2A,C). Similarly, 72 h after transfection, the combination of doxorubicin or vinblastine treatment with survivin silencing greatly potentiated the drug cytotoxic effect (Figure 2B,D). At this time point, doxorubicin treatment promoted about 40% of cell death, while its combination with survivin silencing achieved 60% reduction in cell viability (Figure 2 B). Compared to vinblastine treatment (40% cell death), the effect of previously transfecting cells with anti-survivin siRNAs resulted in an additional 30% of cell death, promoting a 70% reduction in cell viability (Figure 2 D). Parallel experiments performed with mut siRNA resulted in a smaller, although still significant, effect on cell death. Altogether, these results suggest that survivin silencing causes an increase in the susceptibility of A549 cells to doxorubicin and vinblastine treatment.

In the case of HeLa cells, combination of survivin silencing with doxorubicin or vinblastine greatly improved cytotoxicity, increasing cell death by 30−45% (Figure 3). The combination of survivin silencing and treatment with doxorubicin or vinblastine resulted, in all cases, in a significant enhancement of the cytotoxic potential of the drugs. However, in some cases, the reduction in cell viability was statistically comparable to that obtained following survivin silencing alone (Figure 3A,B). Although some extent of cell death was observed after transfection with mut siRNAs followed by incubation with doxorubicin or vinblastine, this effect

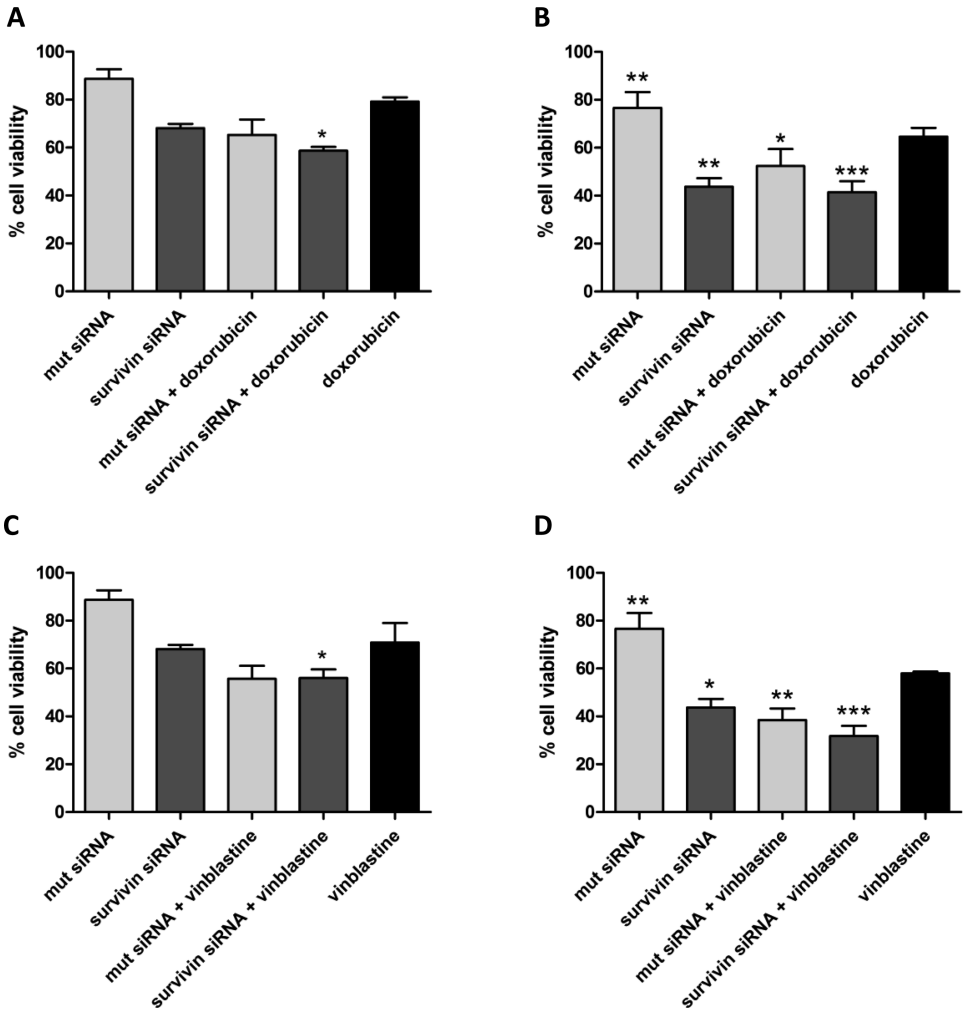

Figure 2. Effect of chemotherapeutic agents on the viability of A549 cells transfected with siRNAs targeting survivin and control siRNAs. A549 cells were incubated with siRNA/Lipofectamine 2000 complexes, at a final siRNA concentration of 50 nM, for 4 h at 37 °C. Twenty-four hours after transfection, cells were incubated with 1 μM doxorubicin or 0.1 μM vinblastine, for 4 h at 37 °C. Cell viability was evaluated by the Alamar Blue assay 48 (A and C) and 72 h (B and D) post-transfection. Cell viability is expressed as the percentage of control (nontreated cells). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to drug treatment of nontransfected cells.

was always significantly smaller than that observed with the corresponding treatment with anti-survivin siRNAs, suggesting that, as observed for A549 cells, survivin silencing renders HeLa cells more sensitive to treatment with doxorubicin and vinblastine.

In addition, the effect of different drug concentrations was studied for the combination that resulted in the highest levels of cell death, which involved survivin silencing followed by vinblastine treatment in HeLa cells (Supplementary Figure 2 in the Supporting Information). It was observed that survivin silencing increases the sensitivity of HeLa cells to vinblastine, thus reducing its IC_{50}, this effect being more evident for low concentrations of this drug.

Effect of the Combination of Chemotherapeutic Agents with Survivin Silencing on Caspase 3/7 Activation in Human Cancer Cells. In order to gain insight into the mechanisms by which survivin silencing enhanced drug cytotoxicity, the activity of effector caspases 3 and 7, crucial components of the apoptotic cell death, was determined.

Figure 4 presents the results obtained for caspase activity in A549 and HeLa cells relative to those obtained after treatment of nontransfected cells with doxorubicin (Figure 4A,C) or vinblastine (Figure 4B,D). Comparing the results obtained for cells that were transfected but not exposed to drug treatment, it is clear that survivin silencing results in much higher levels of caspase activation than equivalent treatment using nonsilencing siRNAs. It is important to note that caspase activity in doxorubicin-treated cells was very low, in both cell lines, being comparable to that obtained for nontreated cells (Figure 4A, C). On the other hand, vinblastine-treated cells presented high levels of caspase activity even in the absence of transfection (Figure 4B,D).

Combination of the survivin silencing and treatment with doxorubicin (Figure 4A,C) resulted in lower levels of caspase activity as compared to those obtained for cells treated only with anti-survivin siRNAs. This is intriguing, since cells exposed to the two different proapoptotic stimuli would be expected to present levels of caspase activation comparable to the ones obtained for the strongest stimulus alone. Therefore, caspase activity of cells treated with anti-survivin siRNAs and doxorubicin should be similar to that obtained upon transfection with anti-survivin

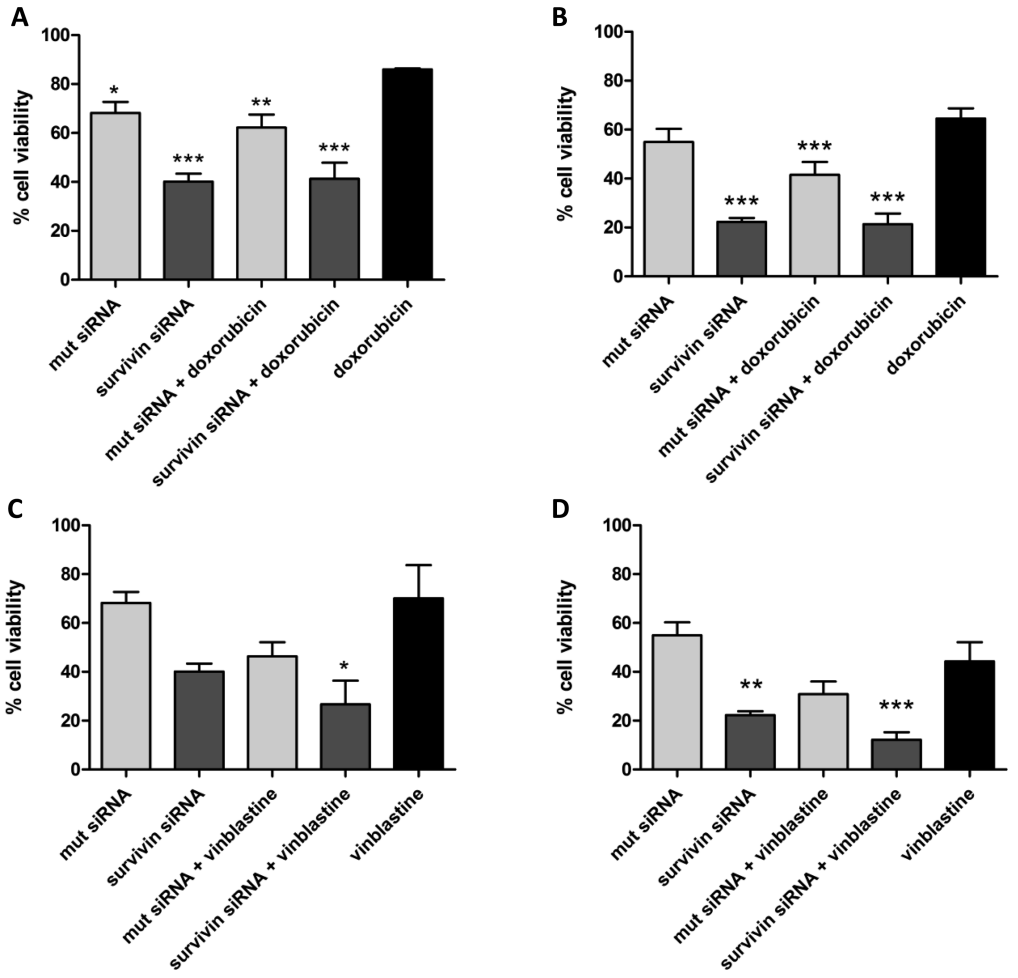

Figure 3. Effect of chemotherapeutic agents on the viability of HeLa cells transfected with siRNAs targeting survivin and control siRNAs. HeLa cells were incubated with siRNA/Lipofectamine 2000 complexes, at a final siRNA concentration of 50 nM, for 4 h at 37 °C. Twenty-four hours after transfection, cells were incubated with 1 μM doxorubicin or 0.025 μM vinblastine, for 4 h at 37 °C. Cell viability was evaluated by the Alamar Blue assay 48 (A and C) and 72 h (B and D) post-transfection. Cell viability is expressed as the percentage of control (nontreated cells). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to drug treatment of nontransfected cells.

siRNAs without drug treatment, and the same should happen for equivalent situations in which cells were transfected with mut siRNAs.

On the other hand, combination of survivin silencing with vinblastine treatment (Figure 4B,D) resulted in an enhancement of caspase activity. In the case of A549 cells, however, a comparable enhancement of caspase activation was observed when cells were transfected with nonsilencing siRNAs, suggesting that nontarget effects or vector toxicity may be potentiating cell susceptibility to vinblastine (Figure 4 B). In HeLa cells, activation of caspases 3 and 7 was comparable in all conditions involving cell treatment with vinblastine, except when cells were previously transfected with anti-survivin siRNAs. In this case, higher values of apoptotic activity were achieved (Figure 4 D), showing that survivin silencing plays an active role in promoting caspase activation.

Effect of the Combination of Doxorubicin with Survivin Silencing on Promoting Apoptosis in Human Cancer Cells. To further explore the events involved in the cytotoxicity mediated by the combination of survivin silencing and drug treatment, assessment of PS asymmetry, which is related to early apoptotic events, was determined (Figure 5A,B). After annexin V and PI staining, cells were analyzed in a dual parametric dot plot (as illustrated in Figure 5C), allowing the separation of the whole cell population in viable cells, early apoptotic cells and late apoptotic cells or necrotic cells. Treatment with Lipofectamine 2000 per se reduced the percentage of viable cells to 60% and 50%, for A549 and HeLa cells, respectively (Figure 5), which may be an indication that some of the nonspecific cytotoxicity observed upon delivery of control siRNAs was caused by the siRNA delivery vector. This hypothesis is further supported by the observation that the cytotoxic effects produced by treating the cells with Lipofectamine 2000 or control siRNA/Lipofectamine complexes were of the same order of magnitude (Figure 5A,B). In contrast, cell treatment with anti-survivin siRNA/Lipofectamine complexes resulted in a clear reduction in the percentage of viable cells, together with a significant increase in the number of late apoptotic cells, as compared to mut siRNA/Lipofectamine complexes (Figure 5A,B). Treatment of nontransfected cells with doxorubicin (bar 4) resulted in a reduction of 50−55% in cell viability, and a similar distribution of cells in the dot plots was observed when cells were incubated with Lipofectamine prior to treatment with doxorubicin. Transfection with control siRNA/Lipofectamine complexes before incubation with doxorubicin

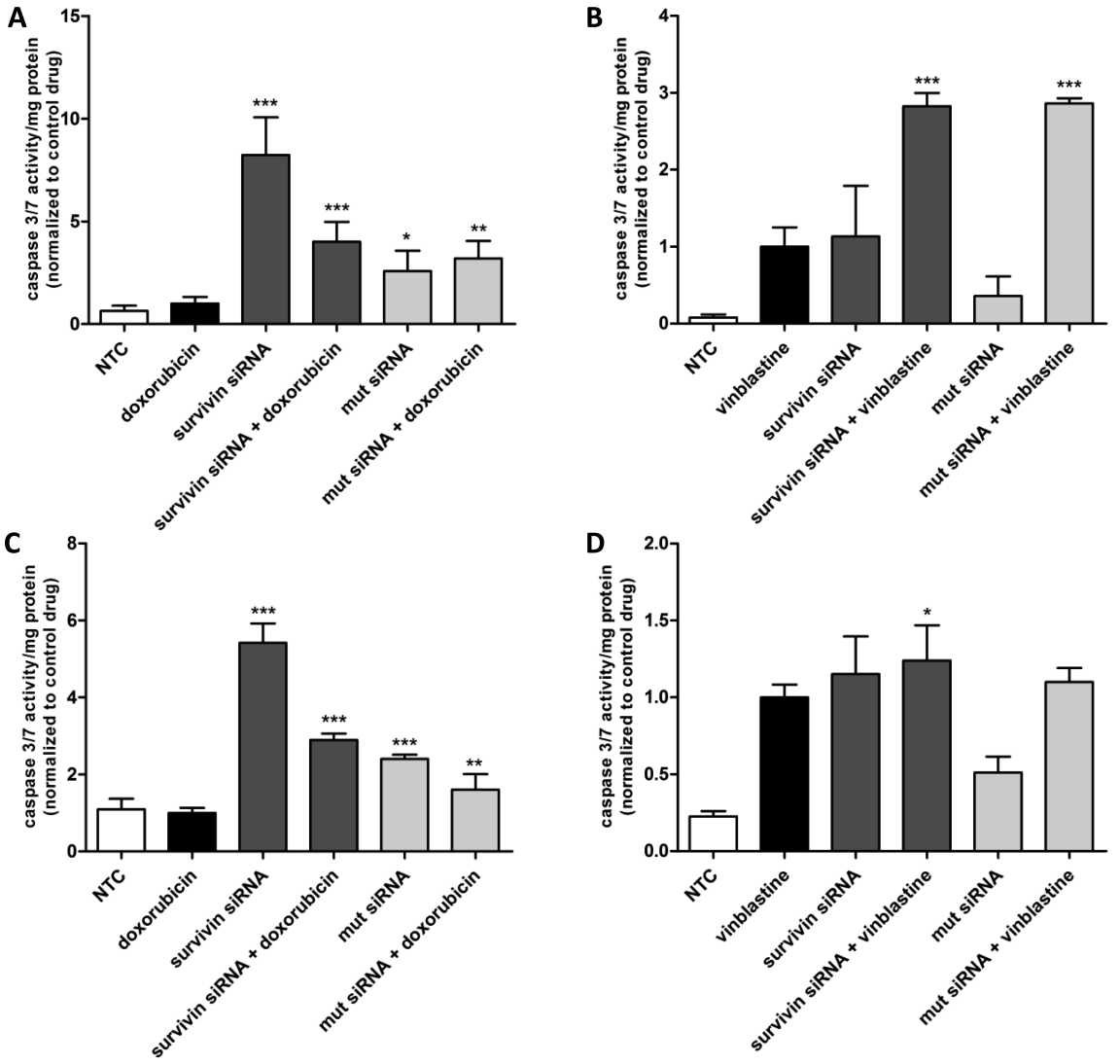

Figure 4. Effect of chemotherapeutic agents on caspase 3/7 activation in cells transfected with siRNAs targeting survivin and control siRNAs. A549 (A and B) and HeLa (C and D) cells were incubated with siRNA/Lipofectamine 2000 complexes, at a final siRNA concentration of 50 nM, for 4 h at 37 °C. Twenty-four hours after transfection, cells were incubated with 1 μM doxorubicin, 0.025 μM vinblastine (HeLa cells), 0.1 μM vinblastine (A549 cells) or HBS, for 4 h at 37 °C. Caspase 3/7 activity was measured 48 h post-transfection in cell extracts by assessing the capacity to catalyze the cleavage of Ac-DEVD-AMC and release the AMC fluorochrome. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to drug treatment of nontransfected cells. Results for nontreated cells (NTC) are shown as a control.

showed slightly higher percentage of late apoptotic cells and a reduction in the amount of viable cells, when comparing to cells that were only treated with doxorubicin. Finally, transfection with survivin siRNA/Lipofectamine complexes followed by treatment with doxorubicin showed the lowest percentage of viable cells, and the consequent higher amounts of early and late apoptotic cells. Thus, combination of both treatments greatly enhanced the cytotoxic effect of doxorubicin, although some improvement was also observed when cells were previously transfected with mut siRNAs, which is in agreement with what was observed in cell viability studies, as assessed by the Alamar Blue assay.

DISCUSSION

It has been recognized that evasion of apoptosis is a hallmark of cancer, and therefore its restoration using RNAi technology to target key antiapoptotic proteins expressed by cancer cells represents an important therapeutic approach.[2,29] Moreover, since apoptosis plays a critical role in the cytotoxic activity of most chemotherapeutic drugs and radiation therapy, the anti-apoptotic proteins overexpressed in tumors, such as members of the IAP family, may constitute the source of chemo- and/or radioresistance.[29,30] Despite the recent advances concerning siRNA delivery to target cells, achieving efficient levels of cellular uptake and long-term stability while avoiding unspecific effects are challenges that need to be met, so that siRNA therapeutics can reach their full potential as a clinically relevant option.[29,31−33] In addition, tumors are often polygenic and present heterogeneous cell populations, possibly expressing different levels of the various pro- and antiapoptotic proteins.[33,34] Therefore, combination of different treatments is expected to improve efficacy, patient management and quality of life. In this context, the combination of RNA-mediated gene silencing with a conventional

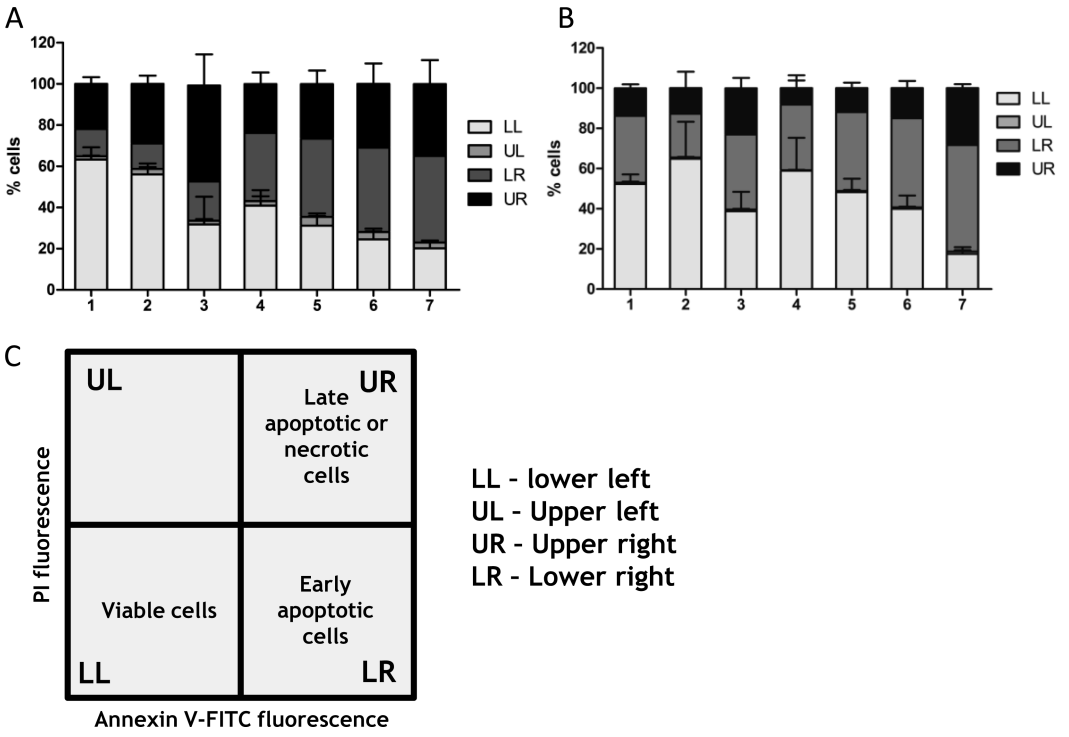

Figure 5. Effect of doxorubicin on promoting apoptosis of cells transfected with siRNAs targeting survivin and control siRNAs. HeLa (A) and A549 (B) cells were incubated with siRNA/Lipofectamine 2000 complexes for 4 h at 37 °C. Twenty-four hours after transfection, cells were incubated with doxorubicin (at ~IC_{50} dose) or HBS, for 4 h at 37 °C. Apoptotic cells were detected through annexin V and PI staining 72 h post-transfection. Each condition was analyzed in a histogram which displays two parameters, annexin V-FITC and PI, as represented in C. The dual parametric dot plots show the viable cell population in the lower left quadrant (annexin V-negative/PI-negative), the early apoptotic cells in the lower right quadrant (annexin V-positive/PI-negative), and the late apoptotic cells in the upper right quadrant (annexin V-positive/PI-positive). The different conditions of cell treatment, represented by numbers below the bars in the graphs, were the following: 1, Lipofectamine; 2, mut siRNA/Lipofectamine; 3, survivin siRNA/Lipofectamine; 4, doxorubicin; 5, Lipofectamine + doxorubicin; 6, mut siRNA/Lipofectamine + doxorubicin; 7, survivin siRNA/Lipofectamine + doxorubicin.

therapy may constitute a promising strategy to achieve complete elimination of cancer cells.[33]

In the present study, we evaluated the efficacy of a strategy that combines cancer cell treatment using a chemotherapeutic agent (doxorubicin or vinblastine) with an RNAi approach targeting the antiapoptotic protein survivin. Initial studies demonstrated that treating A549, HeLa and MCF-7 cells with 50 nM anti-survivin siRNAs, using Lipofectamine 2000 as the delivery vector, resulted in a pronounced decrease of survivin protein and mRNA levels (Figure 1D−F). However, the reduction in survivin levels was translated into a significant decrease in viability only for A549 and HeLa cells (Figure 1A−C). The different sensitivity of different cell lines to the knockdown of the same protein may be explained by the concept of "oncogene addiction".[34] Since cancer cells accumulate multiple genetic, epigenetic, and chromosomal abnormalities over time, it has been postulated that they may become highly dependent on the activity of a single oncogene or oncogenic pathway for maintaining the malignant phenotype and cell survival.[34] Moreover, this "oncogene addiction" seems to frequently differ among different types of cancer, and also among subsets of the same type of cancer.[34] If, on one hand, these findings support the idea that targeting a single specific oncogene can inhibit cancer cell growth and translate to improved survival rates, on the other hand, it may happen that targeting a single oncogene will not have equivalent effects in different types of cancer and sometimes not even in different subsets of the same type of cancer. Finally, in some tumors or tumoral cell lines, the effect of inhibiting one oncogene can be overridden by overexpression of other genes of the same family. Therefore, the effect of survivin silencing can be masked, in some cell lines, by the expression of other antiapoptotic proteins, such as XIAP or Bcl-2.

A significant reduction in the viability of A549 cells (55%) and HeLa cells (80%) was obtained 72 h after treatment with anti-survivin siRNAs delivered by Lipofectamine 2000 (Figure 1A,B). Importantly, cell death determined 48 h after survivin silencing was already significantly higher than that observed with control siRNAs delivered by the same formulation, in both A549 and HeLa cells (Figure 1A,B). However, the small decrease in cell viability observed for nonsilencing siRNAs suggests the occurrence of nonspecific effects of siRNAs or inherent toxicity of the delivery vector. In fact, it is known that exposure of cells to exogenous molecules, including nucleic acids and the delivery vector, has the potential to disturb normal cellular function.[35] It has been suggested that 21 base-pair or longer siRNAs can trigger a nonspecific effect by upregulating interferon responsive genes, an effect that is independent of their ability to mediate sequence specific inhibition of gene expression.[29,35] It is important to mention that when using synthetic siRNAs, which access the RNAi machinery downstream of Dicer, the processing steps that usually determine specificity and efficacy of RNAi when Dicer acts against larger dsRNA molecules, through a proofreading mechanism that protects against the generation of siRNA sequences that might result in the silencing of endogenous genes, do not occur.[29,35]

Chemo- or radioresistance strongly limit the success of cancer therapy, and the involvement of antiapoptotic proteins in preventing cell death has been demonstrated.[36] Some in vitro and in vivo studies showed that survivin downregulation could sensitize human tumor cells of different origins to conventional chemotherapeutic drugs, including paclitaxel, etoposide, cisplatin, topotecan, doxorubicin and geldanamycin.[23,24,26,37−41] XIAP expression levels have also been shown to influence the resistance of cancer cells to the chemotherapeutic drugs, cisplatin, doxorubicin and paclitaxel, in a cell specific manner.[42] Interestingly, exposure of cells to some of these drugs and irradiation has been shown to influence survivin expression, and this effect seems to be dependent on the cell line, and on the p53 gene status of the cells.[23,25,43] Our study aimed at testing the effect of survivin silencing in different human cancer cell lines (A549, HeLa and MCF-7 cells) on their sensitivity to chemotherapeutic agents of different classes, namely, doxorubicin and vinblastine. With this approach we wanted to assess whether survivin overexpression on tumor cells could constitute a mechanism of drug resistance transversal to different types of cancer and independent of the drug classes. Doxorubicin is an anthracycline antibiotic, used in the treatment of various malignancies, which binds to topoisomerase II and allows it to break DNA, but blocks its ability to rejoin the cleaved strands, leading to potentially lethal DNA breaks.[44,45] However, it also causes acute and chronic cardiotoxicity, which limits its clinical application.[44] Thus, a reduction in the amount of administered drug to produce the desired cytotoxic effect would be highly advantageous. Vinblastine is a microtubule inhibitor which is used for treating several types of cancers, including Hodgkin's lymphoma, non-small cell lung cancer, breast cancer, head and neck cancer, and testicular cancer, alone or in combination with other drugs.[46,47] Vinblastine inhibits microtubule polymerization by binding to the region involved in tubulin dimer attachment, suppressing the microtubule dynamics and causing the arrest of cells at the G2/M transition and cell death by apoptosis.[46] Since survivin has a prominent role in regulation of cell division, involving regulation of microtubule dynamics as well as a physical association with Aurora B and Borealin, which targets the chromosomal passenger complex to the kinetochore, allowing the correct completion of cytokinesis,[21,48−50] its expression is expected to some extent to counteract the cytotoxic action of vinblastine. Survivin downregulation should thus allow a reduction of the necessary dose of vinblastine to achieve cancer cell death, thereby reducing therapy-induced side effects.

As discussed above, studies involving the MCF-7 cell line were stopped after we observed that no significant cytotoxic effect could be detected after survivin silencing. Both HeLa and A549 cells exhibited a certain degree of resistance to doxorubicin, as concluded from the observation that approximately 40% of cell death was obtained over a concentration range between 0.5 and 5 μM (Supplementary Figure 1 in the Supporting Information). However, cell response to vinblastine followed a more common profile, and therefore vinblastine concentrations close to the IC_{50} value, 0.1 μM for A549 and 0.025 μM for HeLa cells, were selected for the subsequent experiments on the combination with survivin silencing. The effects of the different treatments on cell viability have shown that survivin knockdown in A549 and HeLa cells potentiated the cytotoxic activity of both doxorubicin and vinblastine, which was more pronounced 72 h after siRNA transfection (Figures 2 and 3). For A549 cells, the doxorubicin concentration that resulted in 40% cell death was able to promote 60% cell death when administered to cells that were pretreated with anti-survivin siRNAs, and the vinblastine concentration that resulted in 40% cell death when used alone could promote 70% cell death when administered to cells pretreated with survivin siRNAs. In the case of HeLa cells, the enhancement of the drug effects was even more pronounced. In these cells, the doxorubicin concentration that resulted in 40% cell death caused 80% cell death when administered to cells that were pretreated with anti-survivin siRNAs, while under these latter conditions, a vinblastine concentration that resulted in 55% cell death when used alone led to 90% cell death. Similarly to what has been found when comparing the cytotoxic effects of anti-survivin siRNA and control siRNA treatments in the absence of any drug, nonspecific effects related to siRNA delivery into the target cells were observed. Although higher levels of cell death were achieved in HeLa cells, the off-target effects of transfection were also more evident in these cells. However, the differences obtained in cell viability between equivalent conditions but using survivin specific or control siRNAs were considerable, and, therefore, it can be concluded that survivin silencing greatly enhances the susceptibility of A549 and HeLa cells to both doxorubicin and vinblastine, thus constituting a valuable approach for antitumor therapy.

Effector caspases 3 and 7 are crucial mediators of apoptosis, being essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, but may also function at the stage when commitment to loss of cell viability is made.[51,52] Although survivin has been shown to bind specifically to the effector caspases 3 and 7, but not to the proximal initiator protease caspase 8,[13] the exact mechanism by which survivin inhibits apoptosis is still unclear. In fact, conflicting results have been obtained regarding the interaction of survivin with caspase 3.[11,53−56] Most likely, survivin interferes with apoptosis through mechanisms that, although culminating in their activation, do not involve a direct interaction with effector caspases. Inhibition of caspase-9, association with XIAP and interaction with SMAC/DIABLO displacing other bound IAPs constitute examples of some of those mechanisms.[11,22,57−59] In this regard, the enzymatic activity of caspase 3 and 7 was assessed in order to unravel the effects of survivin silencing and the combination of this approach with chemotherapeutic drugs on target cells. However, it should be noted that the effect on cell death promoted by the survivin silencing could also be mediated by caspase-independent mechanisms, since survivin has also been shown to inhibit caspase-independent apoptosis.[60] The very low levels of caspase activation observed in both A549 and HeLa cells following doxorubicin treatment (Figure 4A,C) are in agreement with the findings from IC_{50} determination experiments, which revealed that both cell lines presented some resistance to doxorubicin (Supplementary Figure 1 in the Supporting Information). Quite surprisingly, however, caspase activity of cells treated with both survivin siRNA/Lipofectamine complexes and doxorubicin was lower than that of cells treated with equivalent survivin siRNA/Lipofectamine complexes and no drug. In the absence of a synergistic or additive effect, we would expect to obtain the same level of caspase activation for either of the treatments per se. A possible explanation could be that doxorubicin treatment is inhibiting the apoptotic triggering that was observed following survivin silencing in both cell lines, most likely through the activation of antiapoptotic pathways as cellular response to the effects of this drug (Figure 4A,C).

Results from parallel experiments with vinblastine showed that drug or anti-survivin siRNA treatments led to comparable levels of

caspase activation, which were higher than those obtained for cells treated with control siRNAs (Figure 4B,D). When the siRNA-based approach was combined with vinblastine treatment, a significant increase in caspase activation was observed in both cell lines following treatment with anti-survivin siRNA/Lipofectamine complexes (Figure 4B,D). However, no difference could be detected in the apoptosis induction in A549 cells transfected with anti-survivin or control siRNAs, indicating that the observed effect is not specifically related to the survivin knockdown.

The effects on apoptosis resulting from the combination of the survivin silencing approach with doxorubicin treatment were further investigated by annexin V/PI staining and flow cytometry analysis. The results obtained from these studies revealed that treatment with only Lipofectamine 2000 or control siRNA/Lipofectamine complexes reduced the percentage of viable cells to 60% and 50%, for A549 and HeLa cells, respectively (Figure 5), which could mean that, in fact, some of the nonspecific cytotoxicity observed upon delivery of control siRNAs may be caused by the siRNA delivery vector. In contrast, results obtained for cells treated with anti-survivin siRNA/Lipofectamine complexes showed a clear reduction in the percentage of viable cells, together with a significant increase in the number of late apoptotic cells, when compared to those obtained using mut siRNAs (Figure 5A,B), while treatment of nontransfected cells with doxorubicin resulted in a reduction of 50−55% of viable cells. Although transfection with control siRNA/Lipofectamine complexes before incubation with doxorubicin resulted in slightly higher percentage of late apoptotic cells and reduction in the amount of viable cells, when comparing to cells that were only treated with doxorubicin, it was the transfection with anti-survivin siRNA/Lipofectamine complexes followed by treatment with doxorubicin that resulted in the lowest percentage of viable cells, and consequently in higher amounts of early and late apoptotic cells. Together, these findings support the hypothesis that survivin silencing contributes to the reversion of resistance of A549 and HeLa cells to doxorubicin.

Overall, our results demonstrate that by silencing survivin in cancer cells, higher levels of cytotoxicity can be achieved using lower doses of conventional chemotherapeutic drugs. Most importantly, this enhancement occurred even when a drug, such as doxorubicin, to which cells were resistant, was used. Despite the great therapeutic potential of this strategy, issues related to nonspecific immune stimulation, off-target interference and efficiency of in vivo intracellular delivery of siRNAs should be taken into account. Development of biocompatible siRNA delivery systems able to mediate efficient and specific internalization of the nucleic acids into the target tumor cells would significantly increase the clinical interest in this approach.

In summary, we have demonstrated that, at least in two types of cancer cells, survivin silencing promotes the cytotoxic effect of conventional chemotherapeutic agents, which can be of great importance as an effective therapeutic strategy against different forms of cancer. While the exact mechanism by which this effect occurs has not been completely elucidated, it seems to involve restoration of apoptosis, although not always being associated with an enhancement of effector caspase activity.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary Figures 1 (effect of doxorubicin and vinblastine on cell proliferation) and 2 (effect of survivin knockdown on the sensitivity of HeLa cells to vinblastine treatment). This material is available free of charge via the Internet at http://pubs.acs.org.

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